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An Experimental Study on the Function of the Ovarian Germinal Epithelium

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by

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LIFE

Frances Ann Kovarik was born in Chicago, Illinois, July 25, 1939.

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INTRODUCTION

Extensive research has been done on the germ cell problem in vertebrates. These investigations can be divided into two major groups. One is concerned with the origin of the primordial germ cells. The other investigates the possible role of the germinal epithelium in the formation of definitive ova.

The origin and movement of the primordial germ cells has been investigated (see reviews by Brambell, 1956; Mintz, 1960; and Franchii, Mandl, and Zuckerman, 1962). Using cytological criteria, Witschi (1948) described the origin of primordial germ cells in the human embryo from the endoderm of the yolk sac and their subsequent migration into the germinal ridges. In 1954, Chiquoine, utilizing the Gormori technique for alkaline phosphatase, identified and traced the movements of the primordial germ cells in mice. His findings corroborated in all essential details those of Witschii, but he believed these cells to originate from the splanchopleure of the yolk sac. Mintz (1959), following a study of mice, confirmed Chiquoine's findings. She further supported the view that the primordial germ cells are the exclusive source of definitive ova. The extra-gonadal origin of the primordial germ cells has been conclusively demonstrated.

There are two theories regarding the source of the definitive ova. One suggests their origin is formed from the primordial germ

cells. The other considers that they are transformed from the germinal epithelial cells.

The purpose of this investigation is to determine the role of the germinal epithelium in albino rats with special reference to its alleged ability to form definitive ova. A labeling technique was employed by which germinal epithelial cells can be traced from their site of origin into the ovary.

A similar investigation was completed by Latta and Pederson (1944) and Jones (1949) in which the ovarian epithelial cells were labeled with India ink. However, these investigators used different methods and consequently their results were not in agreement. Latta and Pederson concluded that definitive ova arise from the germinal epithelium. Jones, on the other hand, considered that the sole source of definitive ova is the primordial germ cells.

There was a major technical difficulty associated with Latta and Pederson's procedure. They used injections into the periovarian sac as the means of introducing the labeling dye to the ovarian surface; there exists the possibility of the penetration of the syringe needle into the ovarian tissue. Thus, the ovarian stroma could be directly stained. A different type of technical difficulty was associated with the procedure used by Jones. Following an intraperitoneal injection of India ink, the ovarian tissue phagocytized the dye particles. Thus, the dye particles

were taken up, not only by the ovary but by many other phagocytic cells present in the peritoneal cavity.

To avoid the difficulty associated with Latta and Pederson's technique, the present study employed a modified labeling procedure in which suspended dye particles were topically applied to the ovarian surface with cotton saturated with the staining preparation. Also, our technique allowed maximum exposure of the dye particles to the epithelial layer of the ovary. This implies an even distribution of the dye particles on the ovarian surface and the subsequent phagocytosis of most of these particles by the epithelial cells of the ovary.

REVIEW OF LITERATURE

As indicated in the Introduction, this investigation deals with that phase of the germ cell problem having to do with the origin of definitive ova, especially the question of whether or not the germinal epithelium is potentially capable of forming definitive ova. The problem has been extensively reviewed by Everett (1945), Brambell (1956), Zuckerman (1951 and 1960), and Franchii, Mandl, and Zuckerman (1962).

Most early investigators believed that the germinal epithelium could give rise to definitive ova. This conclusion was based solely on histological observations of the ovary. Allen (1923) studied ovaries of normal and unilateral ovarectomized mice, and found a cyclic proliferation of the germinal epithelium. He concluded that definitive ova may arise from these proliferations in the cortex of the adult ovary during each normal estrus.

Kingery (1917), from a histological study of a series of mouse ovaries, reached the conclusion that follicle cells and definitive ova originated from the germinal epithelium. In 1941, Schmidt and Hoffman, by means of intraperitoneal injections of colchicine in guinea pigs, observed many dividing cells in the germinal epithelium. They believed that these proliferating cells were the germinal epithelial cells, not the oocytes. Some of the former cells, in

turn, give rise to the oocytes.

Dawson (1951), following a histological study of polyovular follicles in rats, proposed that follicle cells may have the potential to differentiate into ova if the follicular cells rapidly increase in size. Sneider (1940) made a study of the ovaries of kittens and rats, and observed that new ova were continually produced from the germinal epithelial cells during oogenetic cycles. She based her conclusions on the following: 1) there was an increase in the number of mitosis in the germinal epithelium when new ova appeared beneath its surface; 2) cells with characteristics of oocytes were seen to transform into oocytes in the epithelial cell layer; and 3) meitotic prophases were seen to occur in regular succession from the germinal epithelium into the medullary region of the ovary.

Allen and Creadrick (1937) studied the effect which an injection of colchicine had on prepubertal mice and normal mature rats during estrus. They observed mitoses occurring in the epithelial cells of the rats and mice, believing, therefore, that the germinal epithelial cells do give rise to ova. Similarly, Slater and Dornfeld (1945), studying the histologic changes in the rat ovaries of prepubertal animals, observed a proliferation of the germinal epithelium. They suggested some of the germinal epithelial cells could transform into ova as well as follicle cells.

Vincent and Dornfeld (1948), using a histochemical technique, studied the sites where Desoxyribonucleic Acids (DNA) and Ribonucleic Acids (RNA) were located in the prepubertal rats. They observed areas of proliferation from the germinal epithelium. These areas were restricted to zones in the hilar region of the ovary and had a high concentration of RNA. They suggested the possibility of oocytes being differentiated from the RNA-rich epithelial cells. In 1955, Essenberg, Norowitz, Davidson, and Ryder traced the germ cells from the germinal epithelium until they were fully differentiated ova. In addition to size as a morphological characteristic, the presence of alkaline phosphatase was used as a criterion of oocytes in human embryos.

Employing a totally different technique, Latta and Pederson (1944) injected India ink into the perioverian space in rats. Subsequently, dye particles were observed as inclusions in epithelial cells, follicle cells, and germ cells in various stages of maturation. The results were interpreted to mean that the germinal epithelial cells had phagocytized the dye particles while they were a part of the covering layer of the ovary. They concluded that the germinal epithelium is capable of giving rise to definitive ova.

In 1938, Essenberg and Garwacki studied the ovaries of embryonic chickens as well as young chicks. They failed to see any primordial

germ cells entering the developing gonad, but observed six stages in the differentiation of a germinal epithelial cell into a definitive ovum, prior to the vasculization of the ovary. Thus, they claimed that definitive ova can originate from the germinal epithelium.

In recent years, there has been increasing evidence which supports the alternate theory that definitive ova are derived solely from the primordial germ cells. Everett (1943), after studying irradiated rat ovaries, suggested that ova were formed from primordial germ cells. In his experiments, an intact and healthy germinal epithelial layer had two types of cells: one, of somatic origin and the other, endodermal or germinal. Irradiation apparently destroyed the latter cells without affecting the somatic elements of the ovarian epithelium.

Moore and Wang (1947) removed the germinal epithelium by applying salicylic acid topically to the ovarian surface of the rat, cat, guinea pig, and opossum. Their findings suggest that ova are not necessarily formed from the germinal epithelium, at least for as long as a year postoperatively. Their work was corroborated by the investigation of Mandl and Zuckerman (1950) in the rat, following applications of tannic acid and calcium alignate gauze to the ovarian surface. They also showed that the total number of oocytes in ovaries, which had been denuded, were not significantly

different from that of normal rats, and the total number of follicular cells in the treated animals was only slightly lower than that of normal untreated animals of the same age.

In another experiment, Mandl and Zuckerman (1951) injected carbolic acid into the ovarian bursa of rats. They found that carbolic acid was readily diffusible into the ovarian tissue and caused extensive damage to the cells located deep in the ovarian cortex. As a result, the underlying tissue was involuted while the germinal epithelial cells seemed to withstand the effect of the chemical treatment. The germinal epithelium increased in number of cells and in the size of cells. However, they did not find any mitotic figures in the epithelial layer, but in many places the epithelial cells appeared to be actively phagocytic. There was no apparent relationship between the number of ova present and the condition of the germinal epithelium; thus, they concluded that the germinal epithelium is not essential for oogenesis in the rat, if one accepts the view that oogenesis is a continuing process in the adult.

Through a comparative morphological study of oogenesis in bovine ovaries, Henricson and Rajakoski (1959) demonstrated that oocytic formation is completed by birth, since the nuclei of all the oocytes have entered the first meiotic prophase in the primordial follicles. Enders (1960), following a histological study on the

ovarian cortex of the adult armadillo, concluded that there is not any neoformation of oocytes from the germinal epithelium.

Jones (1949) studied the function of the germinal epithelium by injecting India ink solution into the peritoneal cavity of rats. Subsequent histological study of the ovarian tissue showed that the germinal epithelium is not a source of definitive ova. It is, however, a source of follicular cells, corpus luteal cells, and other supporting elements of the ovary. In 1961, Wang studied the function of the germinal epithelium by performing autoplastic transplantations of normal and denuded rat ovaries. He found, among other things, that the germinal epithelium was indispensible for the survival of the transplanted ovary.

In 1957, Mintz, using a sterility mutate gene (Wj^1), selectively stained the germ cells and traced them into the developing gonad. She found, in the animals having the mutant gene, there were very few primordial germ cells in the embryo. If the germinal epithelium had the potentiality to form ova, some of its cells could have differentiated into ova, thereby increasing the number of germ cells in the ovary, but this was not the case; the animals with the mutant gene, nevertheless, were sterile due to the small number of germ cells in the adult gonad.

Rudkin and Griech (1962) used intraperitoneal injections of irradiated thymidine into pregnant mice mid-way in gestation to

determine the source of definitive ova. They were able to demonstrate the labeled tritium in oocyte nuclei of the pre and postpartum female offspring. They concluded, therefore, that the primordial germ cells are the sole source of oocytes in the mouse.

MATERIALS AND METHODS

A labeling technique, modified from Latta and Pederson (1944), was used in the present experiment. It was designed to avoid any possibility of introducing the dye suspension into the ovary, and to insure maximal contact between the germinal epithelial cells and the applied dye particles. These two objectives were accomplished respectively by replacing the intraovarian bursa injections with the topical application of the labeling substance, and by using glycerin as a vehicle.

The India ink and carmine particles were filtered through double layered filter paper. Next a micrometer study was made on the labeling particles and they were found to have respective average diameters of 2.8 and 2.1 microns. Each was subsequently suspended in a 50 percent glycerin-physiologic saline solution. The glycerin was used to increase the viscosity, act as an adhesive, and keep the ovarian surface moist during the operation. Female Sprague-Dawley albino rats (four to five weeks old) constituted the experimental animals. In each experiment, one of the ovaries was operated upon and the other was left as a normal control. A separate group of animals was used as experimental controls in which a 50 percent glycerin-physiologic saline solution was topically applied to the ovarian surface.

In all cases, the ovaries were exposed through a dorso-lumbar incision. Epinepherin was topically applied to the mesovarii in order to reduce hemorrhage, thereby insuring a clear operative field. The ovarian bursa was removed. The ovarian surface was bathed with physiological saline. In the experimental animals, the bare ovarian surface was coated several times (5-10 minutes) with the glycerin dye preparation; in the experimental control animals, the same procedure was followed except the 50 percent glycerin-physiologic saline did not contain the dye. At the termination of each operation, a two-layered silk closure of the incision was made.

The experimental and control animals were sacrificed by ether anesthesia at intervals ranging from 30 minutes to 4 months post-operatively. The recovered ovaries were fixed in Bouin's solution, dehydrated, and embedded in paraffin. Then they were serially sectioned at 8 microns. Biebrich scarlet was used as a counter-stain for ovaries labeled with India ink particles while Harris Hematoxylin was used for those ovaries labeled with the carmine particles and for the experimental control animals.

A histological study was made of these sections; special attention was given to the location of the dye particles and the type of cells containing them. A thorough search was made especially for any germ cells (oogonia or oocytes) which might have dye particles in their

cytoplasm. Photomicrographs representative of the significant findings were made for the purpose of illustration.

EXPERIMENTAL RESULTS

Information regarding the number of experimental groups, animals in each group, age at operation, postoperative interval, and age of the animals at autopsy is summarized in Table I. The results to be presently described are based on histological observations made from 63 experimental and 13 experimental control rats. Table II summarizes the histological findings in each experimental group which had been exposed to the carmine-glycerin suspension.

General Observations. We were unable to observe a dye inclusion in any of the oocytes present in the ovaries of the experimental animals. The labeled cells decreased with an increase in time following the operative procedure. Dye inclusions in the interstitial cells and macrophages were most frequently seen. Throughout the experimental groups, labeled macrophages were observed; however, in the later groups they decreased in number. Other cells, which were found to contain the dye particles in the cytoplasm, were germinal epithelial cells, small and large cells of the corpus luteum, follicle cells, and adipose cells.

Upon application of the glycerin-dye preparation to the germinal epithelial cells, there seemed to be a general pattern

by which the dye particles were taken up. The amount of dye inclusions in the hilar region was the greatest while in other areas of the epithelium there were few to no particles observed.

Following the study of the experimental group of animals, it was then found that those ovaries, using India ink as the labeling agent, were more difficult to analyze (Fig. 1). This was because the India ink particles tended to clump together on the surface of the ovary; once phagocytized by the cells, these particles seemed to form one large inclusion within the cells rather than to remain as individual particles.

Experimental Controls. In the control group of animals, the topical application of the glycerin-physiological saline solution had no noticeable effect on the epithelial cells (Fig. 2). However, in the 90 and 120 days postoperative groups, there was an increase in the amount of adipose tissue surrounding the ovary as compared to that of the normal controls of the same age.

30 Minutes Postoperative. In these ovaries, it was impossible to determine the sites where the individual dye particles were situated following phagocytosis by the epithelial cells. The entire epithelial cytoplasm was obscured by the density of the stain which had been applied to the ovarian epithelial cells and the fact that inclusions were located in the infranuclear area of these cells (Fig.3).

One Day Postoperative. The greatest amount of dye particles was found in the germinal epithelial cells of the hilar region of the ovary. Some labeled cells were found in the tunica albugenia; these were the macrophages and interstitial cells. The labeled interstitial cells were located in the second and third subepithelial cell layers (Fig. 4). These macrophages are probably normal inhabitants of the ovarian cortex. The presence of the dye inclusions in the macrophage is not clearly understood presumably because of phagocytosis of dead or atrestic labeled epithelial cells (see Discussion).

Two and Four Day Postoperative. The same general picture was seen in these ovaries as in the one day postoperative group of animals. However, there was an increase in the number of labeled macrophages; these cells were in the subepithelial connective tissue (Fig. 5). At this time it was more apparent that certain areas of the ovarian surface were more highly phagocytic than others, e.g., the hilar region. The enhanced phagocytosis is probably one manifestation of the physiological importance of the hilar region (see Discussion).

Five and Six Days Postoperative. In this group of experimental ovaries, the greatest number of labeled cells was found in the germinal epithelial cells and the macrophages. The

labeled macrophages were in a more interior portion of the ovary than previously observed. There seemed to be an increase in the number of labeled interstitial cells; these cells were seen in the tunica albugenia and in more interior portions of the cortical region.

Ten and Fourteen Days Postoperative. In these ovaries, there seemed to be a decreasing amount of stain in the germinal epithelial cells. Much more of the dye inclusions were found in macrophages.

Twenty Days Postoperative. Most of the dye inclusions were seen in the interstitial cells which were scattered in the cortical region of the ovary (Figs. 6 and 7). Other labeled cells observed at this time were epithelial cells and macrophages.

Thirty Days Postoperative. In these ovaries, the greatest number of cells containing dye particles were still the interstitial type. In one ovary which had been stained with India ink, a dye particle was found in a follicular cell. Other labeled cells observed were of the following types: macrophages and epithelial cells.

Thirty-five and Thirty-eight Days Postoperative. As in the preceding experimental group, the interstitial cells were the most predominately labeled cell types (Figs. 8 and 9). It was observed at this time that the number of labeled epithelial cells

were at a minimum. Macrophages and follicular cells were also seen to possess dye particles in the cytoplasm. At this time there was a noticeable regeneration of the ovarian bursa that had been removed at the time of the operation, close to the hilar region of the ovary.

Forty-five and Forty-Seven Days Postoperative. The predominant type of labeled cells was the interstitial cells. Other cells containing dye inclusions were epithelial cells, macrophages, follicle cells, and adipose cells (Fig. 10). In the region where the periovarian sac was being regenerated, macrophages containing dye particles were located.

Sixty and Sixty-two Days Postoperative. The majority of dye inclusions were found in the interstitial cells. As in the preceding experimental group, the number of dye particles seen within the ovary were markedly decreased from that of the thirty day postoperative group of animals. For the first time, labeled theca external and small corpus luteal cells were seen in the experimental ovaries. As in the other groups, labeled macrophages, epithelial, and adipose cells were observed (Fig. 11).

Seventy-two and Seventy-five Days Postoperative. These ovaries had a great amount of adipose tissue surrounding the experimental ovaries when compared with the normal controls. The periovarian sac was completely regenerated by this time. This

phenomenon of the hypertrophy of the adipose tissue is probably related to the operation itself; it apparently has no significant bearing on the results of the experiments in any way. There was an increase in the number of small corpus luteal cells. Also observed were a few large corpus luteal cells containing the dye inclusions. As previously noted, the amount of dye inclusions was greatly reduced. Other labeled cells seen were: interstitial cells, macrophages, epithelial cells, and adipose cells (Fig. 12).

Ninety Days Postoperative. At this time the labeled cells were found most often in the interstitial type. A few corpus luteal cells, macrophages, and adipose cells were found containing labeled dye particles in the cytoplasm (Figs. 13 and 14).

One Hundred Twenty Days Postoperative. In these animals, only a few dye inclusions were found in the ovary. These dye particles were seen mainly in the macrophages and some large corpus luteal cells. Most of the macrophages were observed in the vicinity of blood vessels in the hilar region of the ovary. A few scattered adipose and interstitial cells were seen to contain the dye inclusions (Fig. 15).

Over-all Significance of the Results. The results of this work present a rather consistent and clear-cut picture with respect to the objectives set forth in the Introduction. As far as the

functions of the germinal epithelium are concerned, the observations made in this study strongly deny the possibility that definitive ova can be derived from germinal epithelial cells; however, a variety of cellular elements of the rat ovary, such as interstitial cells, follicular cells, thecal cells, luteal cells, adipose cells, and macrophages, appear very likely to be derived from the mesothelial cells that cover the ovary. For throughout this experiment, it was shown that: 1) the germinal epithelial cells phagocytized the labeling dye particles in large amounts; 2) the ingested dye was subsequently located in other cell types as the number of the original labeled cells decreased with increasing postoperative periods; and 3) most significantly, throughout the experimental material examined, there was not a single case in which a germ cell (oogonium or oocyte) was seen to contain a dye inclusion. If the interpretation given above for the results obtained is correct, it would mean that the technique used in the present experiments has proved itself adequate for the type of problem for which it was intended.

DISCUSSION

Evaluation of the Validity of the Experimental Procedure

From a study of the experimental controls, it was found that the glycerin and physiologic saline solution was nontoxic to the germinal epithelial cells (Fig. 1). This fact is very important because the problem at hand concerns the function of the epithelial cells. There could be no penetration of the dye directly into the ovary for two reasons, namely, 1) we did not use a syringe needle in the application of the glycerin-dye preparation but instead, cotton saturated with the dye preparation; and 2) through the use of the glycerin, we were able to prolong the fluidity of the dye suspension. Thus, the two objectives of the modified labeling technique were realized. The end result jointly attained by them was freedom from injury to the ovarian surface.

Mechanisms of Dye Penetration

Latta and Pederson (1944), Jones (1949), and Mandl and Zuckerman (1951) found that the germinal epithelium of the ovary is highly phagocytic in nature. It is true that we did not observe the epithelial cells actually engulfing the dye particles, but by inference from the existing literature and consistent observations throughout this investigation, this is the only

mechanism by which the dye particles could have become cellular inclusions in these cells.

Inclusions of Labeling Dye in Other Cell Types

Since only the germinal epithelial cells contained the dye particles at the onset of this experiment, we must consider the means whereby the other labeled cells picked up the particles. One possibility is that some of the epithelial cells could lose the dye particles through atresia and subsequently the macrophages engulfed these dye particles. This is unlikely because this phenomenon was never observed in any of the experimental ovaries. Furthermore, the dye particles were consistently found only in the cytoplasm of these labeled cells.

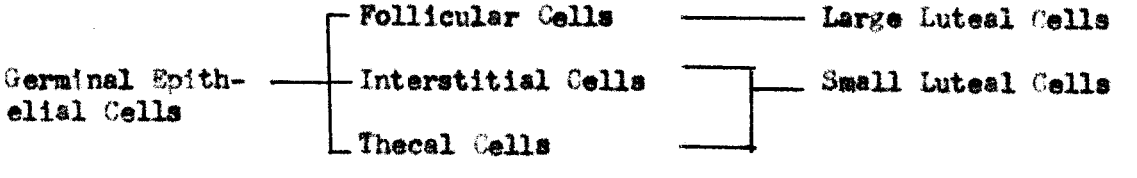
A second possible means of entry of the dye particles into the subepithelial cells is via the inter-epithelial spaces. This is also unlikely because the epithelial cells are highly phagocytic in nature. That is, a particle once coming into contact with an epithelial cell would be immediately ingested. We never observed a single case in which a dye particle was located in the intercellular spaces. Another reason is that the intercellular spaces are not large enough to allow the entry of the dye particles in this manner. Therefore, the only remaining possibility by which these other cellular types of the ovary could acquire the dye is through trans-

formation of a germinal epithelial cell.

Cellular Lineage

The experimental procedure used, therefore, has provided a means by which one can trace the lineage of the germinal epithelial cells. The change of the epithelial cell's location from the surface to the interior of the ovary, can be followed during the course of this experiment (Table II). The other labeled cells all come from differentiated germinal epithelial cells which upon leaving the ovarian surface, changed into different cell types. The other evidence supporting this possibility arises from the fact that none of the cells which were later seen in the cortical region containing dye particles ever came into direct contact with the staining preparation.

A possible lineage chart for the germinal epithelial cells of the ovary is suggested as follows. It is based on one major assumption that the labeling dye particles did not gain entrance into the ovarian cortex, either at the onset of the experiment or did they ever become free from the epithelial cells which originally phagocytized them.



Labeled Macrophages

In this study, it was seen that the experimental dye particles seemed to stimulate the macrophages present in the subepithelial connective tissue. This was also observed by Latta and Pederson (1944) and Mandl and Zuckerman (1951); the former investigators explained the presence of the macrophages by suggesting that they were differentiated from the epithelial cells. However, Mandl and Zuckerman commented that the foreign particles stimulated the already present macrophages in order to augment the means of removing the foreign material. Since the phagocytic nature of the macrophages is established, it is very probable that these mesenchymal cells are already present in the connective tissue and the topical application of the glycerin-dye preparation to the ovarian surface further stimulates them. We did not observe any epithelial cells differentiating into macrophages throughout the material examined.

In the studying of the experimental ovaries of the later groups of animals (30 days postoperatively and on), we noticed a gradual reduction in the density of dye particles present in the ovarian tissue. The means of removal of these dye particles is presumably via the macrophages. During the first four groups of the experimental animals (up to 5-6 days postoperative), it was observed that the macrophages were at first located immediately

beneath the epithelial layer. Later, they were situated more in the interior of the ovary. It appeared that the macrophages migrated into the areas which contained blood vessels, especially in the hilar region. The only plausible interpretation is that the macrophages had phagocytized the dye particles of atretic cells and transported them to the blood vessels and lymphatics, in order to remove the foreign substances from the ovary. Therefore, the number of dye particles remaining in the ovary would continue to decrease with time and those that were left represented dye inclusions of intact cells; these cells underwent the process of cellular lineage as has been discussed above.

Origin of the Interstitial Cells

Since the number of interstitial cells containing dye particles was very large, the origin of these must be accounted for. Kingsbury (1939), Brambell (1956), and Franchii, Mandl and Zuckerman (1962) have reviewed the extensive literature on this topic. The earlier investigators were divided into two major groups: those who supported the view that the interstitial cells were not derived from the germinal epithelium but from connective tissue, thecal cells, or stromal cells. The other group believed that they were differentiated from the germinal epithelium and/or its derivatives.

Rennels (1951) and Dawson and McCabe (1951) found that in rat ovaries there is a dual origin of the interstitial cells. Rennels, following a histochemical and histological study of juvenile and adult rat's ovaries, suggested that there are two types of interstitial cells. The primary type, which is present through the prepubertal phase of development, is "associated with the granulosa outgrowths and ingrowing cords from the germinal epithelium;" this type was found to be the source of estrogen being produced in the ovary at this time. The secondary type of interstitial cells is formed later from the theca interna of atretic follicles. Dawson and McCabe (loc. cit.), from a histochemical study of rat ovaries, believed the secondary type of interstitial cells to be formed from the theca interna of ovulated follicles as well as from atretic follicles. They corroborated Rennel's findings but their results suggested the follicles are responsible for the majority of the primary type of interstitial cells. The results of our investigation suggest the interstitial cells are derived either directly from the germinal epithelium or indirectly from the follicle cells. Since the latter are derived from the germinal epithelium, the interstitial cells are of germinal epithelial origin.

Follicular and Luteal Cells

Our experimental findings agree with the current theory that the follicular cells are derivatives of the germinal epithelium. We based our conclusions on the fact that the dye inclusions were observed in these cells. However, the fact that so few of the follicle cells were actually seen containing the dye remains to be explained.

There are four possible reasons for this. First, in the early postoperative groups of animals, there was a high number of labeled macrophages. The initial high density of the macrophages has probably resulted from the phagocytic nature of these cells upon the atretic epithelial cells as alluded to previously. This very process decreased the number of the initial labeled cells. Second, there is a dilution factor which must be considered. The number of epithelial cells which were previously discussed would be reduced as they give rise to other types of cells. This decreased still further the number of possible follicle cells remaining labeled. Third, the follicle cells, after transformation from the labeled epithelial cells would also become atretic. Consequently, the number of labeled follicle cells observable from the preparations would be even less. Fourth, it is known that following ovulation, the follicle cells transform into corpus luteal cells.

In the later experimental groups, the rats had reached sexual maturity and hence have already ovulated. Therefore, the process of transformation of the follicle cells into luteal cells would have occurred. That explains why in these cases no labeled follicles were seen.

As mentioned, the corpus luteal cells are derived from the follicular and thecal cells. Mossman (1937), following a histological study of the ovary of the pocket gopher, observed that the thecal cells are converted into interstitial cells after ovulation. These interstitial cells may either form the small cells of the corpus luteum or remain interstitial cells. This is in full accord with our observations, namely that the thecal cells formed interstitial as well as small corpus luteal cells. It is, however, to be expected on the grounds just presented that the number of such labeled luteal cells observed would be small.

Definitive Ova

Throughout the study of the slides, dye particles were never found in definitive germ cells during any stage of maturation. This, in itself, does not necessarily constitute proof that any of the germinal epithelial cells which phagocytized the dye never became a germ cell. However, these same results make it highly

improbable that the germinal epithelial cells can differentiate into ova, as contended by many of the early investigators (Allen, 1923; Arai, 1920; and Hargitt, 1930a and 1930b). In view of the fairly large number of experimental animals used, and the wide range in both their age and the length of the postoperative periods used in the experiments, it would seem reasonable to encounter at least one labeled oocyte in our preparations, if one were to believe that germinal epithelial cells are capable of differentiating into ova.

If the germinal epithelial cells had the potentiality to form ova, we might expect to observe at least one case of an oocyte containing the dye; however, this did not occur. We may conclude, therefore, that our results strongly suggest that definitive oocytes are not derived from the germinal epithelium. Mintz (1959) postulated the cause of sterility in mice having a mutant gene (Wj^1) was due to the small number of primordial germ cells situated in these ovaries. Her investigation further supported the theory that the germinal epithelium is not capable of differentiating into ova. Otherwise, these mice would not be sterile for the epithelial cells would make up for the deficit of primordial germ cells. This investigation presents at least supporting evidence for the view that the primordial germ cells are the sole source of definitive ova (Mintz, 1959; Jones, 1949; and Henrickson and Rajakoski, 1959).

Our experimental procedure was effective because we were able to observe the dye particles as cellular inclusions in the epithelial cells, as early as the first day postoperative. At this time, the ovigerous cords of epithelial cells are being formed; it is also the time period, when Latta and Pederson (1944), Butcher (1927), Hargitt (1930a and 1930b), and Long (1940), claimed that they saw the formation of the definitive oocyte surrounded by the follicular nests of cells. Since these investigators based their conclusions upon a histological study of the ovary, this was the logical interpretation for their observations. They believed that the germinal epithelial cells proliferated and gave rise to definitive ova. Our experimental procedure, on the other hand, by virtue of a different technique, enabled us to trace the fate of the labeled cells. Therefore, we arrived at the conclusion that new-formation of oocytes is definitely not one of the functions of the germinal epithelium.

Possible Significance of the Hilar Region

It appears that the hilar region of the ovary is an area of special interest. It is the most proximal end of the ovary; it also marks the transition between the peritoneal mesothelium

and the germinal epithelium. In this region, the blood vessels enter and leave the organ. This study has demonstrated that it contains the highest density of macrophages, which converge toward the blood vessels. In the later experimental ovaries, it was shown that this area sustains the largest amount of adipose tissue around it. From these, and possibly other considerations, it must represent a region of highest metabolic activity.

Vincent and Pernfeld (1948) found the hilar region is the site of RNA-rich cells, and suggested the possibility of the follicles developing as a phenomena of induction. More specifically, they thought that presumably the RNA-rich cells are the oogonia and they can act as an organizer, with RNA as the activator, e.g., the primary stimulating agent (evocator). Through specific organizing influence of the latter, cells originating from the germinal epithelium become organized around the oogonium, forming a unilaminal follicular nest.

Our findings concur with this view with the understanding that the proposed mode of follicular formation brings together cells of two distinctly different sources: the follicle cells derived from the germinal epithelium, and the oogonia from the yolk sac endoderm which have entered the ovary earlier.

SUMMARY AND CONCLUSIONS

The function of the germinal epithelium was investigated by covering the ovarian surface with fine carmine or India ink particles, which were suspended in glycerin. From examination of the experimental controls, the glycerin used was found to be nontoxic to the epithelial cells. Four to five week old Sprague-Dawley albino female rats were used in these experiments; the experimental animals had a postoperative period which ranged from 30 minutes to four months. The recovered ovaries were fixed and stained for detailed histological examination. The following results are based on the total of 63 experimental ovaries so studied.

1. As early as 30 minutes after the application of the dye preparation, most germinal epithelial cells were seen to have phagocytized the particles. The latter were of such a density that the entire cells appeared red, having the nucleus appear heavily stained.

2. In all other experimental ovaries examined, regardless of the length of the postoperative period, dye particles have been observed in a variety of cell types. The dye particles were

invariably seen in the cytoplasm, never in the intercellular spaces.

3. Under the experimental conditions it was considered impossible for the dye particles to gain entry into the ovary through the spaces between the epithelial cells. The only possible means for the dye particles to enter the ovary was via phagocytosis by the epithelial cells.

4. On the basis of these observations, the presence of the dye particles, which were seen later within various cell types, strongly indicate that all these cells have transformed from the labeled epithelial cells.

5. It was ascertained that most cell types of the ovary, except germ cells, were found to contain varying amounts of the dye particles.

6. Since not a single germ cell was ever observed containing the dye particles, the results of this research constitute strong supporting evidence that the germinal epithelial cells are not endowed with the potential to form definitive ova. Our results concur with many recent investigations on this subject that the

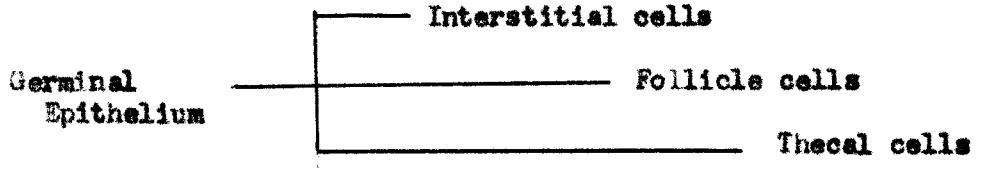
primordial germ cells are the sole source of definitive ova.

7. A gradual disappearance of the originally labeled epithelial cells from the ovarian surface was observed in ovaries with increasingly longer postoperative periods. In ovaries recovered four months postoperatively, only occasional labeled epithelial cells were seen in the original position.

8. It was also observed that the labeled cells shifted from the periphery of the ovary to a more central location. As these cells translocated, they changed into several definitive cell types.

9. During this translocation, the originally labeled cells became scattered in the ovarian cortex. As they mingled with other cellular elements, one gets the impression of a progressive decrease in the density of the labeled cells.

10. Our results indicate that at a given postoperative age, there were various labeled cells of which one type predominated. Assuming that all these cells have been derived from the originally labeled epithelial cells, the various cell types have probably differentiated at different times. This relationship may be expressed as follows:



11. One labeled cell type, which was seen throughout this work was the macrophage. We have no reason to believe that these cells have originated from the germinal epithelium. It is known that these cells are normally present in the ovarian cortex, and are carrying out their normal function of removing the foreign substances. Although not observed in our material, we assume that the macrophages might have phagocytized the dye particles from atretic labeled cells. In support of this view, we have observed the movement of the labeled macrophages converging toward blood vessels.

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Tables and Plates

TABLE I. SUMMARY OF EXPERIMENTS

Exp.* Group	No. of Rats	Age at Operation	Postoperative Interval	Age at Autopsy
1	3	30 days 31 days 37 days	30 min.	30 days 31 days 37 days
2	4	30 days 32 days 34 days 34 days	1 day	31 days 33 days 35 days 35 days
3	5	30 days 31 days 31 days 32 days 35 days	2 and 4 days	33 days 33 days 33 days 34 days 38 days
4	5	30 days 31 days 31 days 35 days 35 days	5 and 6 days	35 days 36 days 37 days 41 days 41 days
5	5	30 days 31 days 35 days 35 days 35 days	10 and 14 days	44 days 47 days 45 days 45 days 45 days

* For each experimental group one rat was operated on as an experimental control.

TABLE I CONTINUED

Exp. Group*	No. of Rats	Age at Operation	Postoperative Interval	Age at Autopsy
6	5	30 days 31 days 35 days 36 days 37 days	20 days	50 days 51 days 55 days 56 days 57 days
7	5	30 days 32 days 35 days 36 days 37 days	30 days	60 days 62 days 65 days 66 days 67 days
8	5	30 days 31 days 32 days 35 days 38 days	35 and 38 days	68 days 66 days 68 days 73 days 73 days
9	5	31 days 31 days 35 days 38 days 40 days	45 and 47 days	76 days 78 days 82 days 85 days 77 days
10	5	30 days 32 days 32 days 34 days 35 days	60 and 62 days	90 days 92 days 92 days 94 days 95 days

*For each experimental group one rat was operated on as an experimental control.

TABLE I CONTINUED

Exp. Group	No. of Rats	Age at Operation	Postoperative Interval	Age at Autopsy
11	5	32 days 32 days 37 days 38 days 38 days	74 days	106 days 106 days 111 days 112 days 112 days
12	5	30 days 30 days 30 days 37 days 37 days	90 days	120 days 120 days 120 days 127 days 127 days
13	5	30 days 35 days 35 days 36 days 36 days	120 days	150 days 155 days 155 days 156 days 156 days

*For each experimental group one rat was operated on as an experimental control.

TABLE II

HISTOLOGICAL FINDINGS OF THE LOCATION OF THE CARMINE DYE INCLUSIONS IN THE EXPERIMENTAL OVARIES

Exp. Group	Epithelial Cells	Macrophages	Adipose Cells	Follicular Cells	Interstitial Cells
30 min.	++++++	-	0	0	-
1 day	+++++	+++	0	0	++
2 and 4 day	+++++	++++	0	0	++
5 and 6 days	+++++	+++++	0	0	+++
10 and 14 days	++++	+++++	0	0	++++
20 days	+++	++++	0	0	++++
30 days	+++	+++	0	0	++++
35 and 38 days	+++	+++	0	+	+++++

Scale	-	was not able to determine the location of the inclusions
	0	no dye inclusions present
	+	minimal amount of dye
	++	
	+++	moderate amount of dye
	++++	
	+++++	
	++++++	maximal amount of dye present

TABLE II CONTINUED

Exp. Group	Epith. Cells	Macro-phages	Adipose Cells	Folli-cles	Inter-stitial	Thecal Cells	Corpus Luteum Sm. Cells	Corpus Luteum Lge. Cells
45 and 47 days	++	++	+	+	+++	0	0	0
60 and 62 days	++	+++	++	++	++++	+	+	0
72 and 75 days	++	++	+	0	+++	0	+	+
90 days	+	++	++	0	+++	0	+	+
120 days	+	++	+	0	+	0	0	0

	-	was not able to determine the location of the inclusions
	0	no dye inclusions present
	+	minimal amount of dye
Scale	++	
	+++	moderate amount of dye
	++++	
	+++++	
	++++++	maximal amount of dye present

PLATE I.

Figure 1. Thirty day postoperative experimental ovary. Note the clumping of the India ink particles in the cytoplasm of the cells. Magnification 600x.

Figure 2. Germinal epithelium of a four day post-operative control ovary. Note no effect of the glycerin on the epithelial cells which are in one of the crypts. Magnification 600x.

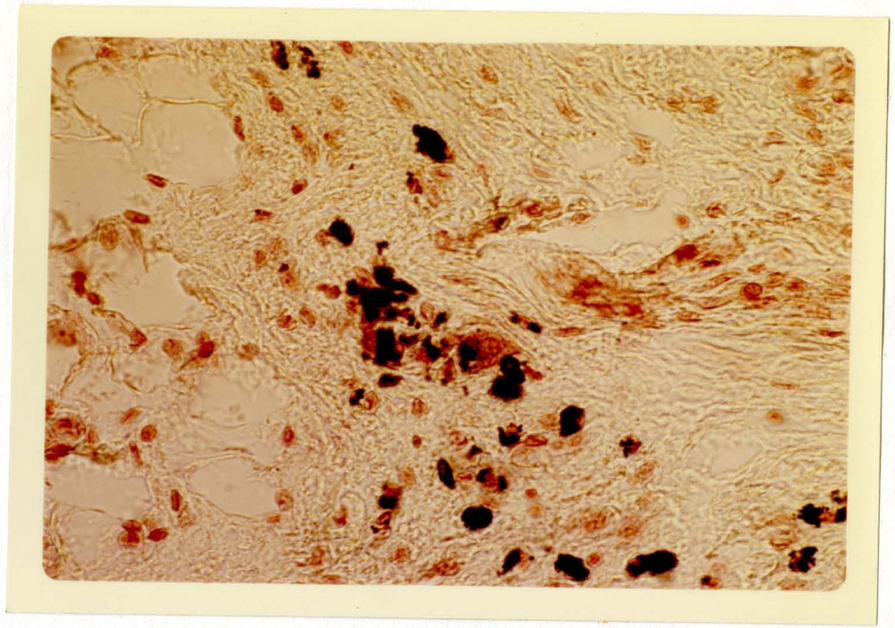

Figure 1.

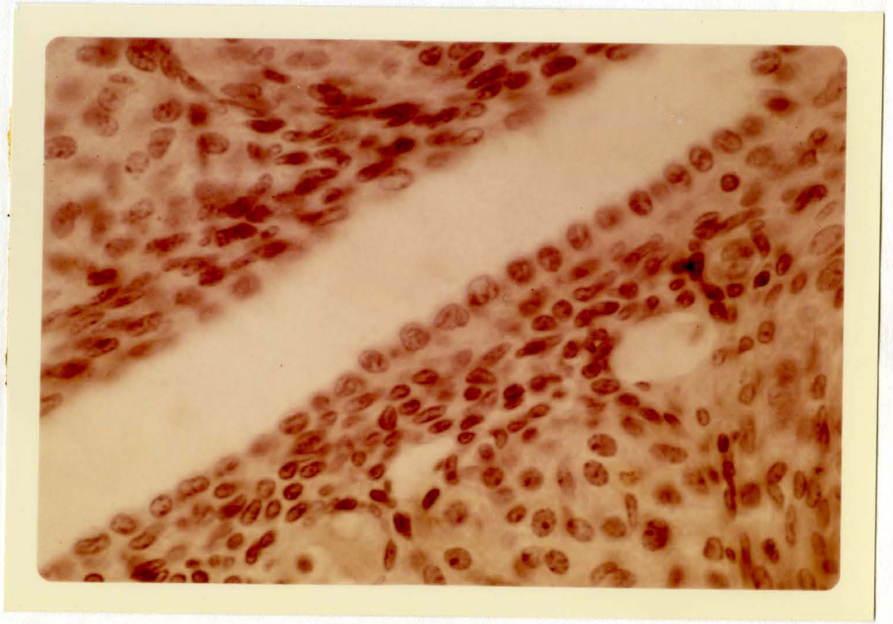

Figure 2.

PLATE II.

Figure 3. Cortical region of a thirty minute postoperative animal. Note the density of the dye particles in the epithelial cells; no individual particles can be observed. Magnification 600x.

Figure 4. Ovary of a one day postoperative experimental animal. Note the presence of the dye inclusions in the cells located in the tunica propria. Magnification 600x.

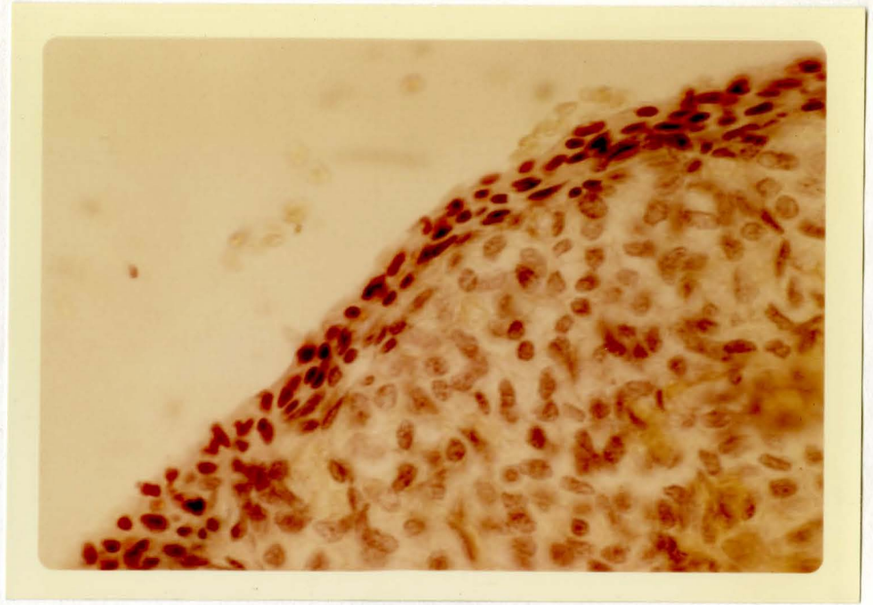

Figure 3.

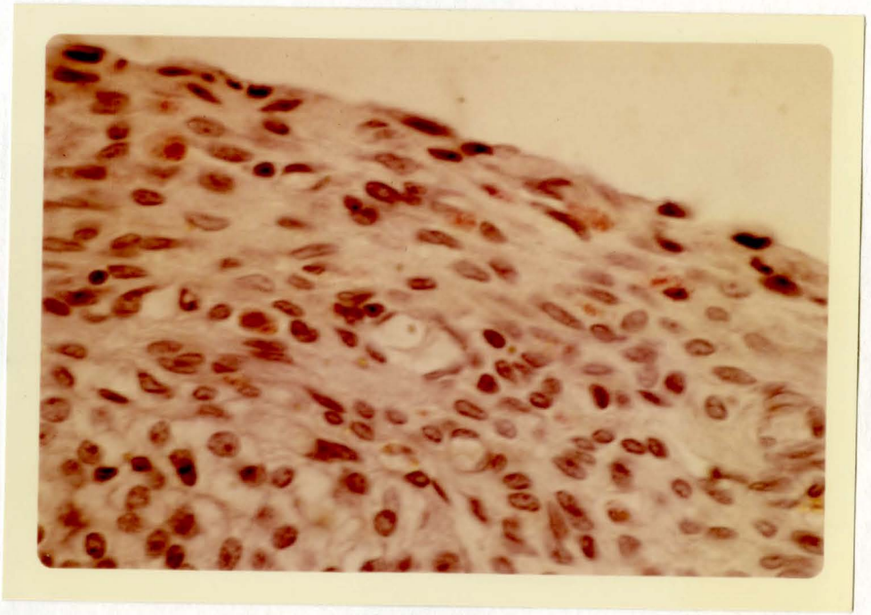

Figure 4.

PLATE III.

Figure 5. Hilar region of a five day postoperative ovary. Note the presence of carmine inclusions in macrophages, tunica albugenia cells, and interstitial cells. Magnification 600x. M - macrophage, T A - tunica albugenia, and I - interstitial cell.

Figure 6. An ovary of a twenty day postoperative experimental animal. Note the density of the macrophages in the hilar region of the ovary. Magnification 600x.

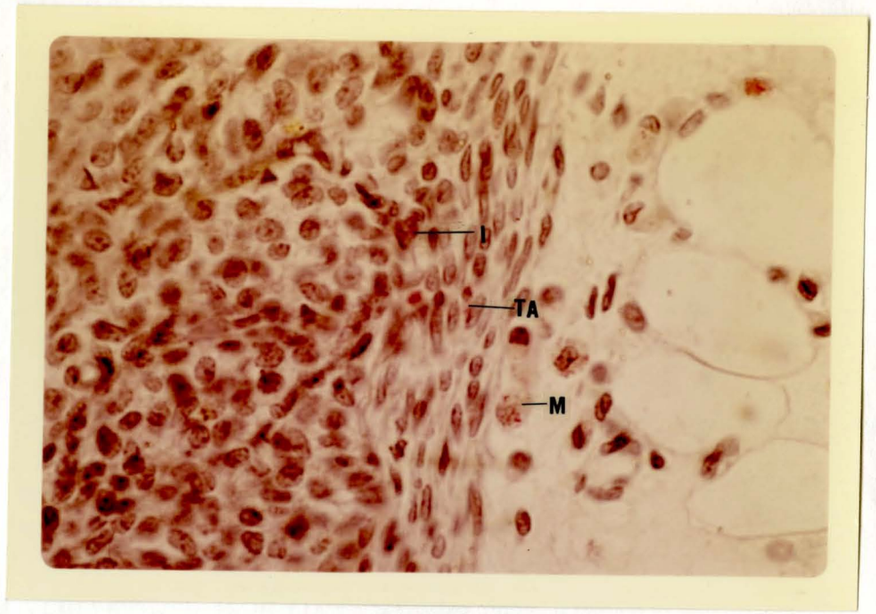

Figure 5.

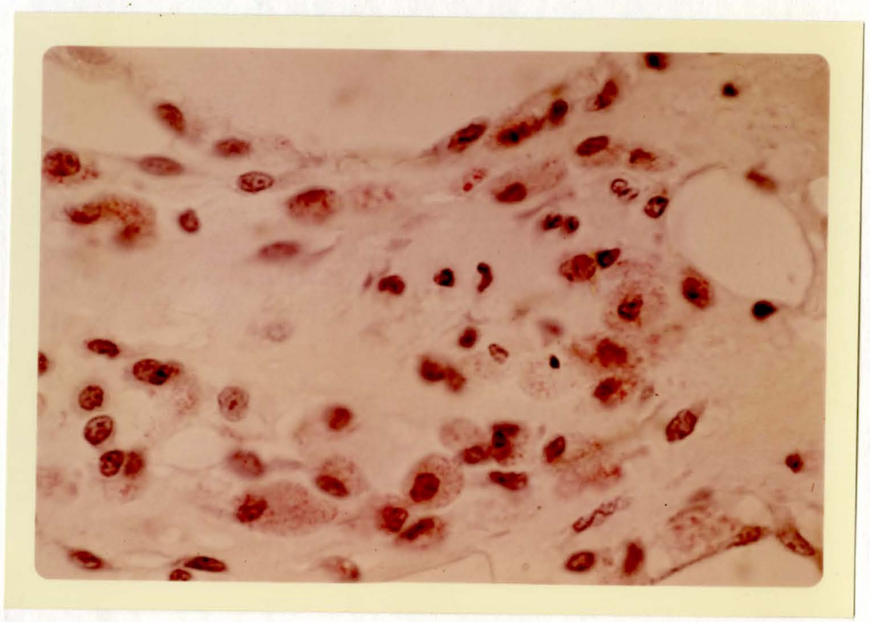

Figure 6.

PLATE IV.

Figure 7. A portion of the cortical region of a twenty day postoperative ovary. Note the dye particles in the interstitial cell. Magnification 1500x.

Figure 8. The cortical region of a thirty-eight day postoperative rat ovary. Note the presence of carmine inclusions in macrophages and epithelial cells in the region of two developing follicles. Magnification 600x. E - epithelial cell and M - macrophage.

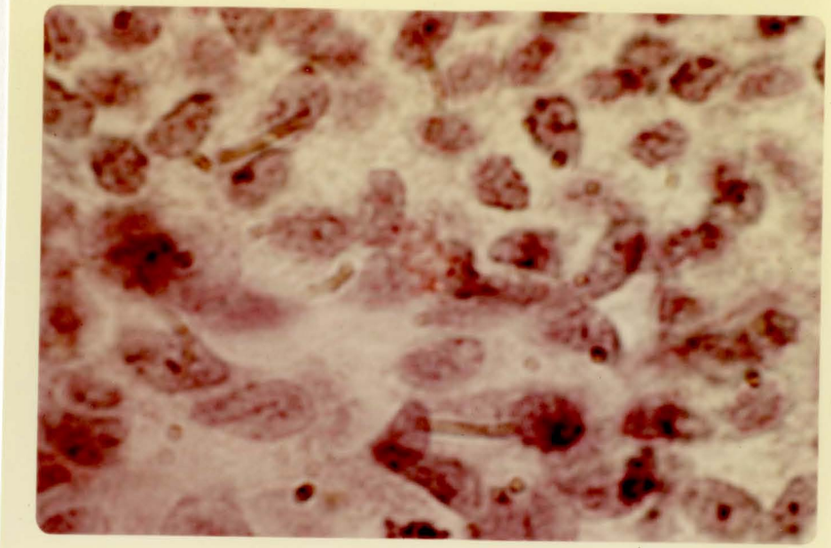

Figure 7.

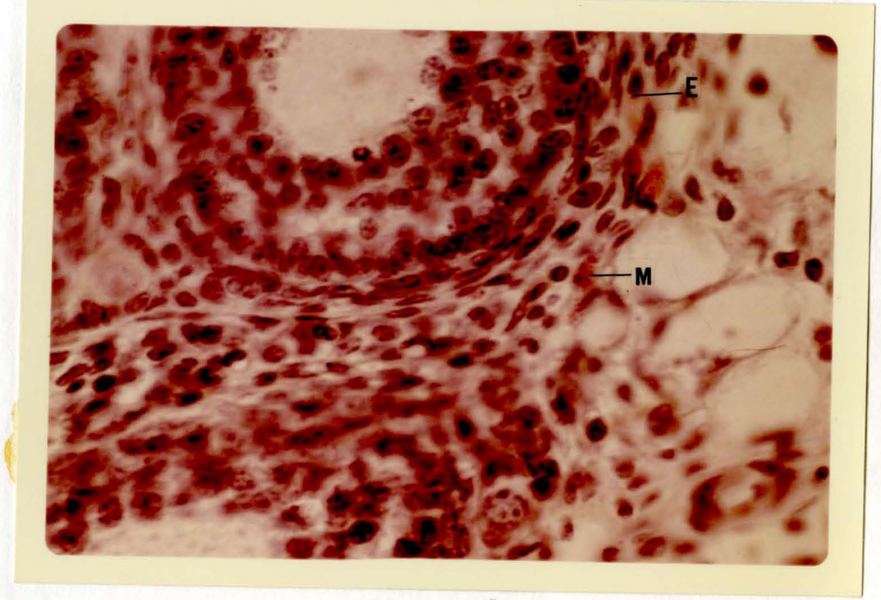

Figure 8.

PLATE V.

Figure 9. An ovary of a thirty-five day postoperative animal, showing a section of one of the epithelial crypts. Note the presence of the light dye particles in two adjacent follicular cells. Magnification 375x. F - follicular cell.

Figure 10. Hilar region of a forty-five day postoperative rat ovary. Note the dye inclusions in adipose cells and macrophages. Magnification 600x. A - adipose cell and M - macrophage.

Figure 9.

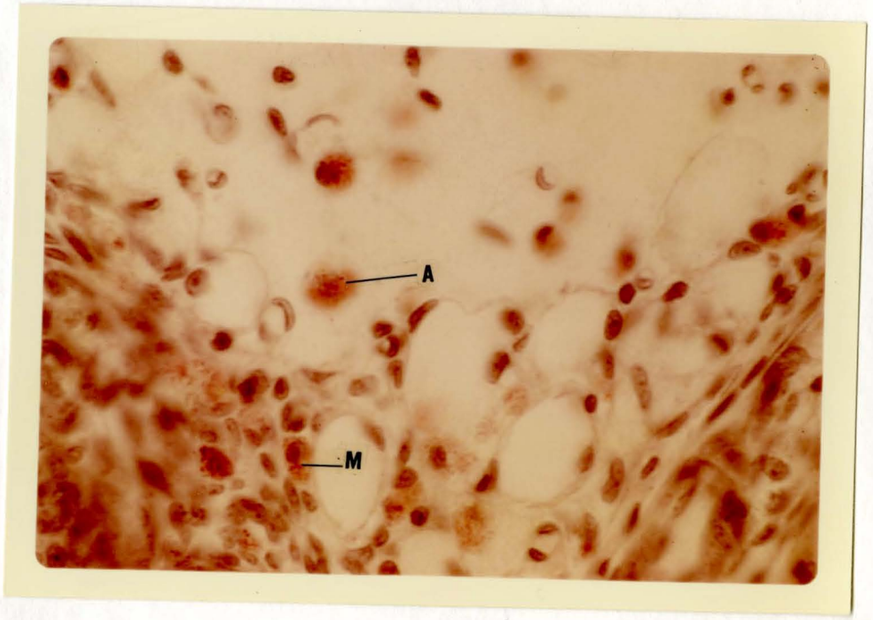

Figure 10.

PLATE VI.

Figure 11. An ovary of a sixty day postoperative rat. Note the dye particles in the theca externa cell and macrophages. Magnification 600x. T E - theca externa cell and M - macrophage.

Figure 12. The cortical region of a seventy-two day postoperative ovary. Note the scattering of the dye particles in the ovary and the clumping effect of the India ink particles. Magnification 300x.

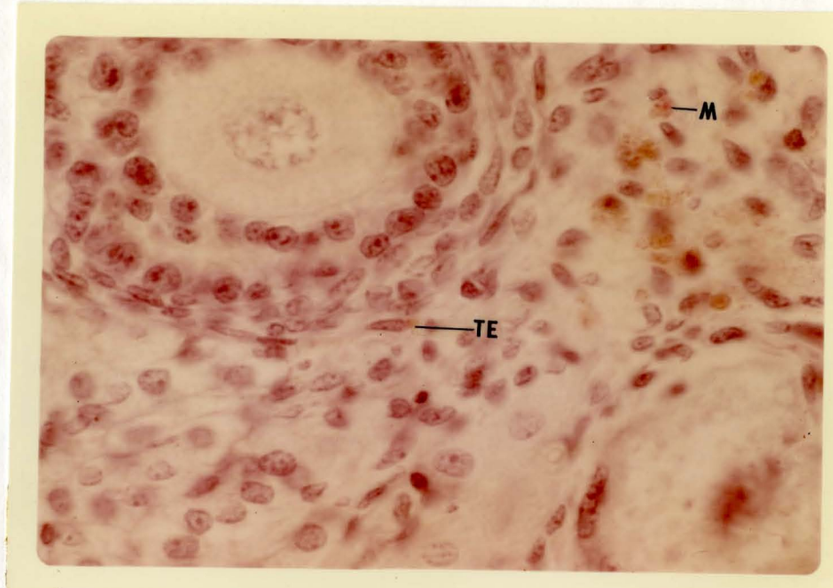

Figure 11.

Figure 12.

PLATE VII.

Figure 13. An ovary of a ninety day postoperative animal. Note the dye inclusions in the corpus luteal cell and the presence of the corpus luteal granules. Magnification 1500x. CL - corpus luteal cell and CG - cytoplasmic granules in a corpus luteal cell.

Figure 14. The hilar region of a ninety day postoperative rat ovary. Note the number of labeled macrophages containing dye inclusions. One of these macrophages has a nucleus and the other does not, due to the level of the section. Magnification 600x.

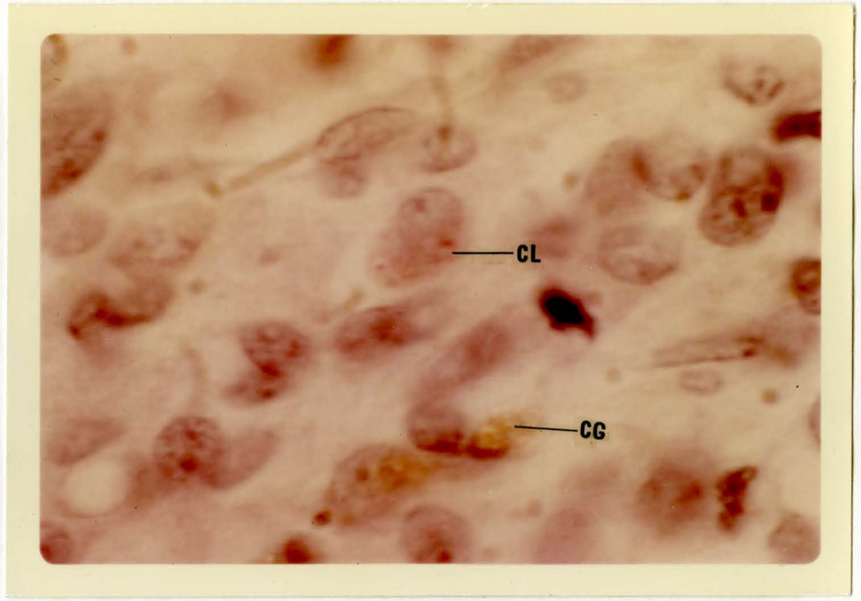

Figure 13.

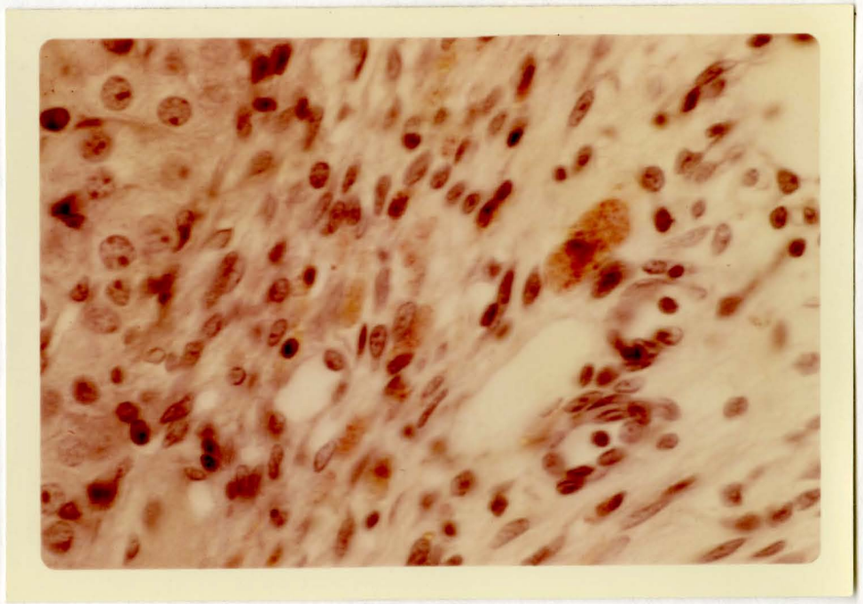

Figure 14.

PLATE VIII.

Figure 15. The hilar region of a one hundred twenty day postoperative ovary. Note the presence of the dye inclusions in the macrophages which are in close association with the capillaries. Magnification 600x.

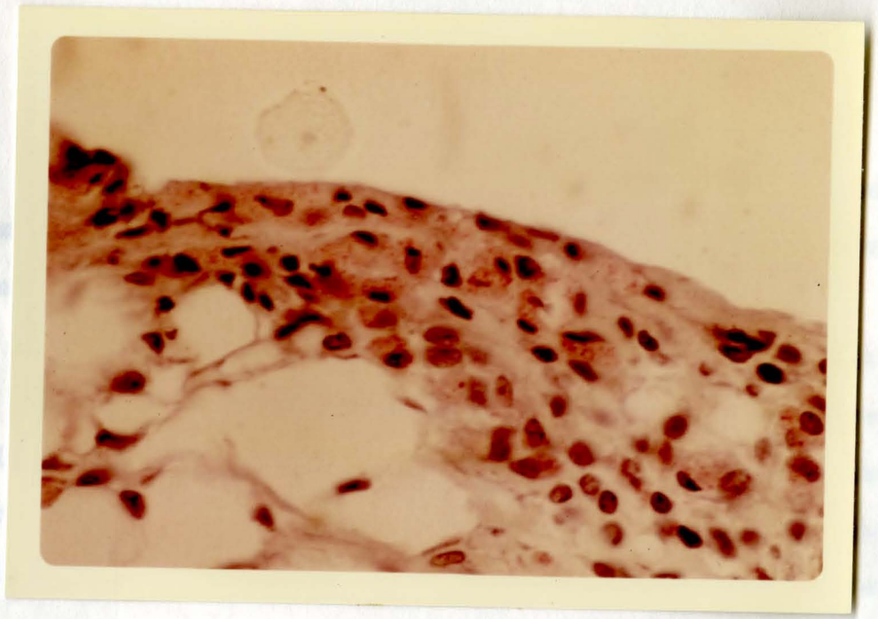

Figure 15.

APPROVAL SHEET

The thesis submitted by Frances A. Kovarik has been read and approved by three members of the Department of Anatomy.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 25, 1964
Date

Harry Wang
Signature of Advisor